

Adenovirus-mediated preproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA^y mice

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Abstract We investigated whether adenovirus-mediated preproinsulin gene transfer into insulin target tissues (adipocytes) ameliorates hyperglycemia in diabetic mice. KKA^y mice, a genetically obese type 2 diabetic animal model, were treated with a single subcutaneous injection of recombinant adenovirus, Adex1CA-human preproinsulin (Adex1CA-pchi), into the epididymal fat pads. pchi mRNA was expressed only in adipose tissue in which mature insulin was produced. Three days after virus injection these mice showed a marked decrease of blood glucose levels (from about 400 to 200 mg/dl), and an intraperitoneal glucose tolerance test revealed the markedly improved glucose tolerance. There was no significant difference in serum insulin levels between control and recombinant adenovirus-treated KKA^y mice. The normalized glucose levels in diabetic mice were maintained for at least 2 weeks after the virus injection. This strategy could provide a novel and, most importantly, a simple and convenient gene therapy for obese type 2 diabetes patients. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Adipocyte; Gene therapy; GLUT4; Insulin; Obesity; Type 2 diabetes

1. Introduction

Type 2 diabetes, which affects more than 100 million people worldwide [1], is caused by a combination of insulin resistance and a declined capacity of pancreatic β cells [2,3]. One possible therapeutic strategy for type 2 diabetes would be to construct insulin production and secretory pathways within non- β cells by transferring the relevant genes and using these cells as a substitute for β cells. It is essential, however, that the production and/or the secretion of insulin in such cells be physiologically regulated. Many research groups have tried to expand or regenerate pancreatic β cells by genetic engineering techniques [4]; however, in most of these trials, a definite way to regulate insulin secretion from those cells was not established [5–8]. In the present study, we took advantage of the

facts that adipocytes characteristically secrete many adipocytokines (e.g. leptin, tumor necrosis factor (TNF)- α , plasminogen activator inhibitor-1) [9] and that the regulated exocytotic pathway of facilitated glucose transporter 4 (GLUT4) can be triggered by insulin stimulation [10,11]. We therefore investigated the effect on hyperglycemia of transferring preproinsulin genes into adipose tissues of obese diabetic KKA^y mice. KKA^y mice have genetically determined obesity and diabetic syndrome [12–14]. These mice show no sexual or seasonal variation in the appearance of glycosuria, but show hyperglycemia and hyperinsulinemia at the age of 5 weeks. The gene therapy procedure reported here showed some success in lowering the blood glucose levels of diabetic KKA^y mice.

2. Materials and methods

2.1. Recombinant adenovirus

Adenovirus containing human preproinsulin (pchi) cDNA (a generous gift from Dr. G.I. Bell, University of Chicago), Adex1CA-pchi, was constructed as described previously [15]. It contains the full length of pchi cDNA under the control of the modified chicken β -action promoter. Adex1w, containing no foreign cDNA, was used as a control.

2.2. Animal treatments

KKA^y mice were obtained from Japan Clea Co. (Tokyo, Japan) and fed ad libitum. Animals used in the experiments were 10–12 weeks old, their average weight was 28.8 ± 4.2 g, and blood glucose levels were >300 mg/dl. Mice were treated with 0.3 ml of 1×10^9 – 5×10^9 pfu/ml of either Adex1CA-pchi or Adex1w (control) by a single subcutaneous injection into the epididymal fat pads. For another series of experiments, Zucker fa/fa rats were obtained from Charles River (Yokohama, Japan). Animals used in these experiments were 12 weeks old and were treated with 2 ml of 1×10^9 pfu/ml of either Adex1CA-pchi or Adex1w (control) as described above. 3 days after injection, blood was drawn from the tail vein to determine glucose concentration.

For the intraperitoneal glucose tolerance test (ipGTT), 6-h-fasted mice or overnight-fasted rats were intraperitoneally challenged with 2 g/kg body weight glucose, and blood samples were drawn at the indicated time point from the tail vein. Mice were killed by decapitation, then testis, intestine, lung, liver, skeletal muscle, cardiac muscle, and epididymal fat pads were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C until use.

2.3. Reverse transcriptase (RT)-polymerase chain reaction (PCR) Southern blot analysis

Total tissue RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method [16]. To detect human insulin cDNA, RT-PCR was performed as described previously [15]. The 345-bp PCR products were run on a 1.2% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P -labeled human insulin cDNA probe as described previously [15].

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GLUT, facilitated glucose transporter; ipGTT, intraperitoneal glucose tolerance test; pchi, human preproinsulin; RT-PCR, reverse transcriptase-polymerase chain reaction

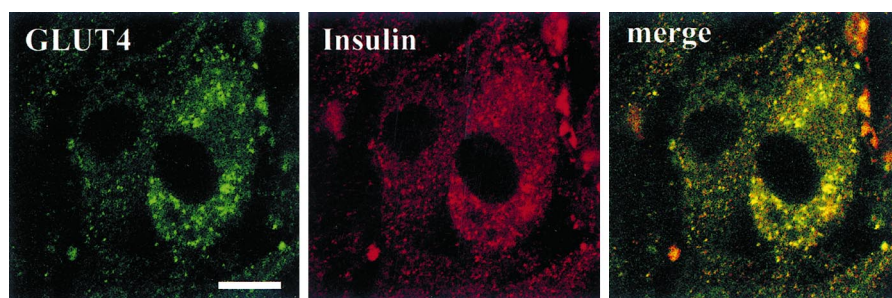


Fig. 1. Colocalization of (pro)insulin and GLUT4 in the same vesicle in a 3T3L1 adipocyte. 3T3L1 adipocytes were infected with Adex1CA-pchi as described in Section 2. The infected cells were immunostained with rabbit polyclonal anti-GLUT4 and/or mouse monoclonal anti-insulin antibodies, followed by appropriate second antibodies (GLUT4: FITC; insulin: rhodamine), and observed by confocal laser scanning microscopy. The yellow color (merge) obtained after overlaying FITC (GLUT4) and rhodamine (insulin) indicates the colocalization of (pro)insulin and GLUT4 in the same vesicle. Bar = 10 μ m.

2.4. Immunoblot analysis

Immunoblotting was performed as described previously [17]. Briefly, total proteins from adipose tissues were extracted by sonication; the proteins were then separated by electrophoresis and transferred to nitrocellulose membrane filters. Filters were incubated with a guinea pig anti-insulin antibody (1:1000 dilution), and the bands were visualized using a chemiluminescence detection system (NEN).

2.5. Immunocytochemistry

3T3L1 cells were grown on coated glass chamber slides (eight wells; Lab-Tek slides, Nunc), and differentiated to adipocytes by incubation in medium containing 10% fetal bovine serum (FBS), 1.7 μ M insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 mM dexamethasone. 3T3L1 adipocytes were infected with Adex1CA-pchi at a multiplicity of infection of 30 for 1 h in 5% FBS Dulbecco's modified Eagle's medium; after changing the medium, the cells were cultured for 48 h and then used for immunocytochemistry. After the slides were washed several times with KRB containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.3 mM Ca gluconate, 4.8 mM NaHCO_3 , 2.2 mM glucose, 10 mM HEPES (pH 7.4), and 0.3% bovine serum albumin, they were fixed with 2% paraformaldehyde. The slides were double-immunostained with rabbit polyclonal anti-GLUT4 (a generous gift from Dr. K. Takata, Gunma University, Gunma, Japan) and/or monoclonal anti-insulin (Sigma) antibodies, followed by appropriate second antibodies, and examined by confocal laser scanning microscopy (Model LSM510, Carl Zeiss Co., Jena, Germany) at the excitation wavelengths of 488 nm for fluorescein isothiocyanate (FITC) and 529 nm for rhodamine with a band-pass filter.

2.6. Insulin measurement

Serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA; MBL, Nagoya, Japan), which reacts with both mouse and human insulin equally. To detect only human insulin in mouse serum, we used a human insulin ELISA assay system with monoclonal anti-human insulin antibody (Dainabot Co., Japan), which showed less than 2% cross-reactivity with mouse insulin (data not shown).

2.7. Statistical analysis

Unless stated otherwise, results are means \pm S.E.M. from at least three different experiments performed independently. Statistical analyses consisted of ANOVA followed by Fisher's test and regression analysis using Statview software (Abacus Concepts, Berkeley, CA, USA).

3. Results and discussion

In the present study, we attempted to take advantage of the characteristic features of adipocytes as endocrine organs [9]. We hypothesized that if an adipocyte itself secretes insulin via a regulated vesicle, such as a GLUT4 vesicle, its insulin receptor could be exposed to high concentrations of released insulin, and, subsequently, the released insulin may amplify

the insulin action through the endogenous insulin receptor in an autocrine manner. Indeed, when we infected the 3T3L1 adipocytes with Adex1CA-pchi, (pro)insulin was colocalized with GLUT4 vesicles (Fig. 1). 3T3L1 adipocytes expressing human insulin were immunostained with both rabbit polyclonal anti-GLUT4 and mouse monoclonal anti-insulin antibodies. Secondary antibodies against rabbit (FITC label) and mouse (rhodamine label) IgG did not cross-react (data not shown). As shown in Fig. 1, the yellow color (Fig. 1, merge) obtained by overlaying the FITC signal (GLUT4) with the rhodamine signal (insulin) indicated the colocalization of the expressed (pro)insulin with endogenous GLUT4 in the same vesicles. Thus, (pro)insulin could be released via the exocytosis of GLUT4 vesicles in response to exogenous insulin stim-

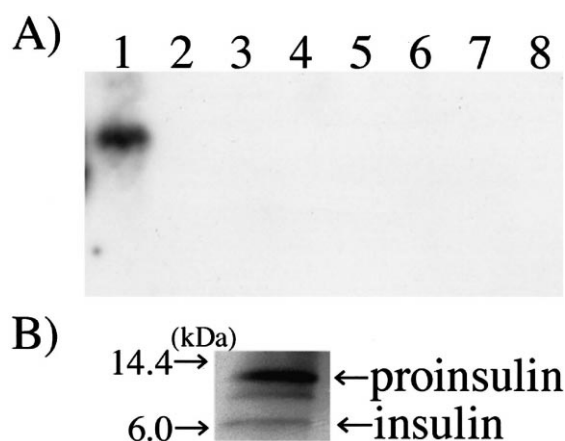


Fig. 2. Preproinsulin mRNA and protein expression in epididymal fat pads of KKA^y mice treated with recombinant adenovirus. KKA^y mice were treated with a single subcutaneous injection of 0.3 ml of 1×10^9 – 5×10^9 pfu/ml Adex1CA-pchi into the epididymal fat pads. A: Preproinsulin mRNA detected by RT-PCR Southern blot analysis. 3 days after the virus injection, epididymal fat pads, testis, liver, lung, intestine, skeletal muscle, and cardiac muscle were removed, and processed for RNA isolation. The cDNAs were reverse-transcribed from total RNAs and subjected to the PCR; PCR products were separated by 1.2% agarose gel electrophoresis, blotted onto a nitrocellulose filter, and hybridized with ^{32}P -labeled human insulin cDNA probe. A representative autoradiograph of three separate experiments is shown: (1) epididymal fat pads; (2) testis; (3) intestine; (4) lung; (5) liver; (6) skeletal muscle; (7) cardiac muscle; (8) negative control. B: Proinsulin and insulin expression in adipose tissues. Total protein was extracted from adipose tissues of recombinant adenovirus-treated KKA^y mice and subjected to immunoblot analysis with guinea pig anti-insulin antibody. Protein bands were detected by a chemiluminescent detection system.

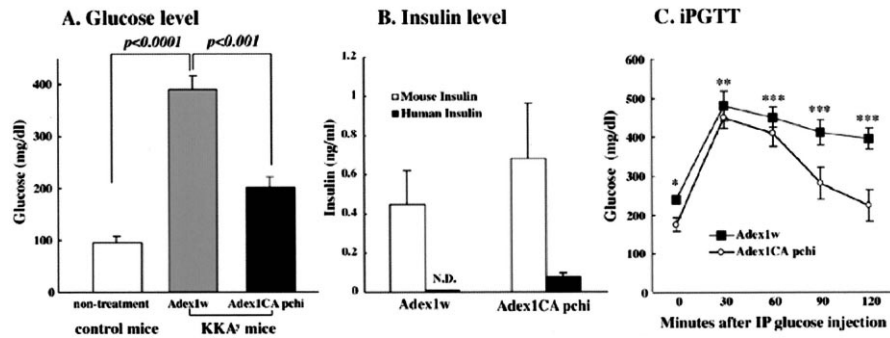


Fig. 3. Blood glucose level, insulin level, and ipGTT of mice treated with recombinant adenovirus. KKA^y mice were fed ad libitum until the ages of 8–12 weeks. These mice were treated with 0.3 ml of 1×10^9 – 5×10^9 pfu/ml of Adex1CA-pchi or Adex1w (control) by a single subcutaneous injection into the region of the epididymal fat. A: Blood glucose levels: random blood samples were taken from tail veins of fed mice 3 days after injection with recombinant adenoviruses. Blood glucose levels were significantly lower in KKA^y mice treated with Adex1CA-pchi than those treated with Adex1w (control mice, $n = 10$; KKA^y mice treated with Adex1w, $n = 6$; KKA^y mice treated with Adex1CA-pchi, $n = 4$). B: Serum insulin levels. Serum insulin levels were measured by ELISA using blood samples taken 3 days after virus injection. Open column shows serum insulin levels containing mouse and human insulin, and solid column shows only human insulin levels measured by human insulin ELISA system. C: ipGTT. After mice were fasted for 6 h, glucose (2 g/kg body weight) was injected intraperitoneally; blood samples were then collected at the indicated time intervals from the tail vein ($n = 4$). * $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$ versus Adex1CA-pchi-treated mice.

ulation by a mechanism in which insulin-receptor signaling causes the movement of GLUT4 vesicles from the intracellular pool to the plasma membrane [18].

We investigated the effect on blood glucose levels of expressing the preproinsulin gene in adipose tissues in KKA^y mice, an animal model for obese type 2 diabetes [12–14]. We infected adipose tissues of KKA^y mice with recombinant adenovirus, Adex1CA-pchi or Adex1w (control), by a single subcutaneous injection into the epididymal fat pads. RT-PCR Southern blot analysis revealed that preproinsulin mRNA was expressed in adipose tissues of the mice treated with Adex1CA-pchi, but not in surrounding tissues such as testis and intestine (Fig. 2A). We also did not observe any mRNA expression in liver, lung, skeletal muscle, or cardiac muscle. Fig. 2B clearly shows the immunoblot protein band of proinsulin and insulin in the adipose tissues. Although proinsulin-converting enzymes PC2 and PC3 are not expressed in adipocytes, furin expressed in adipocytes (data not shown) can process proinsulin to mature insulin, although with low efficiency [19]. The introduction of furin-cleavable consensus sequence into pchi may cause the efficient conversion of proinsulin to insulin in adipose tissue. Thus, in adenovirus-injected adipose tissues of KKA^y mice, pchi gene was expressed and eventually mature insulin was produced.

Three days after adenovirus injection into adipose tissues of KKA^y mice, we measured blood glucose levels in fed animals. As shown in Fig. 3A, KKA^y mice treated with Adex1CA-pchi showed a decrease in blood glucose levels (202 ± 20.4 mg/dl, $P < 0.001$ versus Adex1w [control] group: 391 ± 27 mg/dl). There was no significant change of serum insulin levels between Adex1w- (control) and Adex1CA-pchi-treated KKA^y mice. In addition, the serum level of immunoreactive human insulin (measured by human insulin ELISA) was very low in Adex1CA-pchi-treated KKA^y mice (about 70 pg/ml; Fig. 3B), indicating that the decrease in blood glucose levels is due to an autocrine effect in the adipose tissues. Because adipose tissue is a relatively minor glucose disposal site in the body, however, mechanisms other than the proposed autocrine loop are also possible. The plasma levels of free fatty acids [20], leptin [21,22], TNF- α [23,24], and resistin [25] released from adipose tissues play a role in regulating glucose metabolism,

and the expression of human insulin gene in adipose tissues may affect these parameters. We are currently investigating these possibilities.

Results of the ipGTT showed a gradual decrease in blood glucose levels in Adex1CA-pchi-treated KKA^y mice (Fig. 3C). It is noteworthy that the marked drop of blood glucose levels was observed at 90 and 120 min after glucose loading (90 min: 281 ± 41 versus 412 ± 33 mg/dl, $P < 0.001$; 120 min: 223 ± 41 versus 396 ± 28 mg/dl, $P < 0.001$). Because this system is switched on only after insulin released from pancreatic β cells binds to the endogenous insulin receptor on adipocytes harboring the preproinsulin gene, it cannot reduce the blood glucose level immediately after glucose loading. A potential advantage of this system, however, is that sustained hyperglycemia may be suppressed without causing hypoglycemia, because the secretion of adipocyte insulin is indirectly regulated by blood glucose levels, as mentioned above. When considering the use of this gene therapy in humans, this observation is critical for practical application. An eventual decrease in blood glucose levels as shown by ipGTT indicates that local

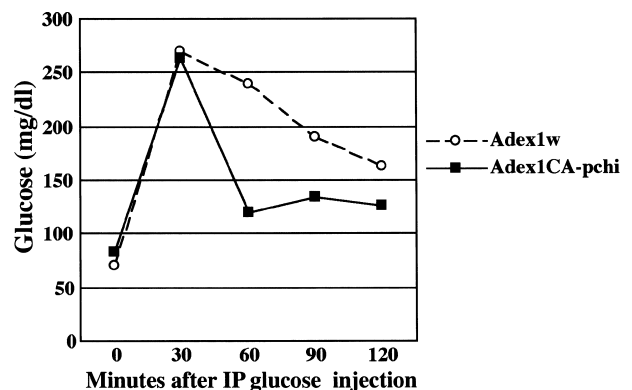


Fig. 4. Glucose tolerance test of Zucker fa/fa rats treated with Adex1CA-pchi. Zucker fa/fa rats (12 weeks old) were treated with 2 ml of 5×10^9 pfu/ml of Adex1CA-pchi or Adex1w (control) by a single subcutaneous injection into the region of the epididymal fat pads. After rats were fasted overnight, ipGTT was performed as described in the legend of Fig. 3. Data represent the mean of two experiments.

exposure of extremely high concentrations of insulin, which is exocytosed via an initial GLUT4 translocation after glucose loading, accelerates the glucose uptake.

Our failure to reduce the rapid increase of glucose level after glucose loading may be due to impaired GLUT4 translocation, which might be caused by GLUT4 down-regulation in adipocytes of diabetic, insulin-resistant mice [26]. It is known that insulin sensitivity of adipose tissue in KKA^y mice decreases with age [13]; however, at the age of 10–12 weeks, the insulin-resistant status in adipose tissue is still incomplete. Abdominal administration of insulin (2 U/kg) markedly decreased the blood glucose levels from 400 to 100–200 mg/dl in KKA^y mice (unpublished results). KKA^y mice (9 weeks old) pretreated with the analog of thiazolidine showed a marked drop of blood glucose levels by insulin administration (0.5 U/kg, i.p.) [27]. Thus, at this age the insulin signal in adipose tissue is expected to be still, but not completely, intact in KKA^y mice. To examine the effect of this procedure on another animal model, we injected 2 ml of Adex1CA-pchi into epididymal fat pads of obese Zucker fa/fa rats. 2 days after virus injection, we performed an ipGTT. Fig. 4 shows that the impaired glucose tolerance observed in fa/fa rats was slightly improved by Adex1CA-pchi treatment.

Finally, we examined the time course of blood glucose levels in Adex1CA-pchi-treated KKA^y mice. The lowered blood glucose levels were maintained for at least 2 weeks after the virus injection. Adex1CA-pchi-treated mice showed lower blood glucose levels (from approximately 400 to about 150 mg/dl) from 2 days after virus treatment until 2 weeks after receiving recombinant adenovirus (Fig. 5). Although some researchers have reported that adenovirus-transferred gene expression was sustained for up to 15 months [28], we found that the blood glucose levels were back to 400 mg/dl only 1 month after virus injection, which is consistent with the disappearance of human insulin gene expression in the virus-injected region (data not shown). We propose that proper glucose levels are maintained via mutual communication between pancreatic β cells and adipocytes (Fig. 6).

Although the precise mechanism underlying this system must still be determined by *in vitro* experiments, which we are currently pursuing, we emphasize the beneficial effects

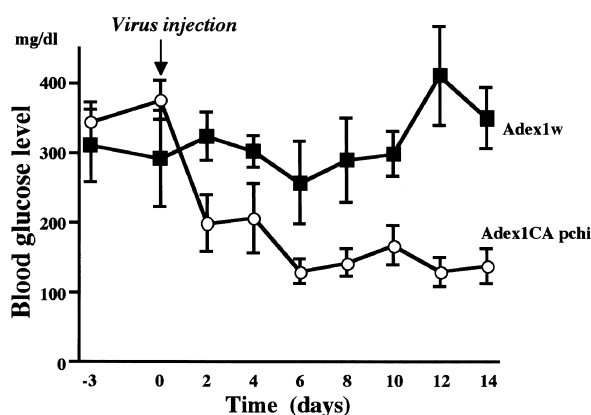


Fig. 5. Ectopic expression of pchi gene in adipocytes of diabetic KKA^y mice ameliorates hyperglycemia for 2 weeks after the virus injection. KKA^y mice were injected with Adex1CA-pchi ($n=4$) or Adex1w (control; $n=3$) as in the legend of Fig. 2, and glucose levels were determined in random blood samples drawn from the tail vein of fed mice.

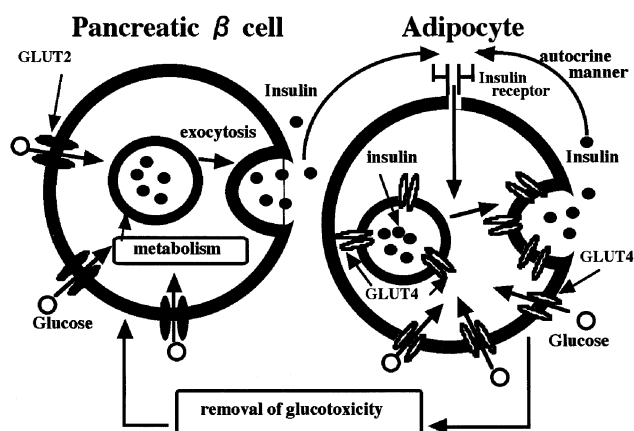


Fig. 6. Scheme of our concept of the mutual communication between pancreatic β cells and adipocytes. Insulin released from pancreatic β cells activates the autocrine system in the adipocytes harboring the preproinsulin gene, which subsequently eliminates the glucotoxicity, followed by the restoration of pancreatic β cell functions.

and advantages of this therapeutic system. This gene therapy is very simple and convenient, particularly for the treatment of obese type 2 diabetes. Obese diabetic patients could easily subcutaneously inject the virus into their abdominal fat, potentially controlling glucose levels.

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